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Transgenic Mouse Models

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14. ABSTRACT There is emerging evidence that transformed stem cells may be the source of human cancers. We felt that transgenic mouse models were ideally suited to examine this question and proposed to conduct marrow transplant experiments to test whether marrow stem cells are the cells of breast cancer origin. Our most significant findings included: 1) the demonstration that stromal cells within the transgenic breast cancers contain significant percentages of tissue macrophages, lymphocytes, fibroblasts, myofibroblasts and endothelial cells, which presumably represent the progeny of cancer-promoting stem cells of donor origin; 2) the demonstration that these stromal cells of donor origin may affect breast cancer progression differently than those of endogenous breast origin; 3) the demonstration that rare cancer-initiating stem cells of donor origin can give rise to breast cancer; and 4) the demonstration that these ectopically-derived stem cells that transform to breast cancer may be different biologically compared to the endogenous breast stem cells that spontaneously transform to breast cancer.					
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INTRODUCTION

Although some human breast cancers are inherited, the majority arise spontaneously. In both inherited as well as acquired breast cancer, clonal cancer development is rare. Even when all breast cells contain inherited BRCA1/2 mutations or are exposed to estrogen/radiation, for example, breast cancer occurs from the progeny of only a single transformed cell (1). The breast does however contain multifocal ductal proliferations. These findings have been interpreted as the multihit pathway of cancer origin, the multifocal proliferations reflecting a first “hit” but the rare cancer emerging from accumulations of additional “hits” (2). But this hypothesis may not be correct. There is emerging evidence that transformed stem cells may be the source of human cancers (3) and the stem cell hypothesis alternatively may account for the rareness of breast cancer. We recently studied 14 patients who had received a different sex donor bone marrow transplant for lymphoma / leukemia who later developed a solid cancer and made a startling observation. In 7 patients (2 breast cancers), based on X/Y chromosomal FISH and confirmatory LOH studies, the solid cancer was of donor origin! (Figure 1). These observations suggested that the donor’s marrow might contain the cell of cancer origin! We felt that this observation/hypothesis needed to be tested experimentally. We felt that genetically engineered breast cancer mouse models were ideally suited to do this and proposed to conduct marrow transplant experiments in these transgenics to test whether marrow stem cells are the cells of breast cancer origin.

BODY

For our first objective, we proposed to introduce “genetically marked” bone marrow derived from tumor-prone mice into either normal healthy or unmarked tumor mice. If our hypothesis was correct, we expected to observe clonal donor breast cancers in either wild type or tumor-prone recipients. For our second objective, we proposed to introduce bone marrow from “genetically marked” healthy mice into unmarked tumor-prone mice. In this case we might expect a decrease in breast cancer incidence in the transgenic mice receiving wild type marrows. In all the groups we monitored tumor onset and genetically assessed the origin of the cancers that arise: donor or recipient.

For our first objective, we used two different murine female bitransgenic breast cancer models (4): 1) the highly penetrant though non-physiologic *MMTV-pymT* / *ROSA26* transgenic where polyomavirus middle T antigen was under the control of the MMTV promoter and in which all of its tissues including bone marrow expressed the lacZ reporter; and 2) the less penetrant but more natural *MMTV-erbB2/neu* / *ROSA26* bitransgenic. Marrow from each of the female bitransgenics harvested by femoral flushing, were tail vein-injected into lethally irradiated normal healthy female mice as well as their respective unmarked transgenic recipients. In these latter recipient groups, if the hyperplasias which were observed were recipient in origin but the clonal mammary carcinomas which emerged were donor in origin it would indicate that the traditional “multihit” hypothesis that has dominated our field for the past several decades may not be correct. Rather it would indicate that clonal cancers arose from bone marrow-derived stem cells and not from multifocal ductal hyperplasias. For our second objective, we used normal marked female *ROSA26* as donors and each of the oncogene transgenics as recipients. Our end point in our second objective was the prevention or a decrease in breast cancer development.

If our hypothesis that the bone marrow contained breast cancer stem cells was correct, we could utilize cell type specific marker analysis (Figure 2A) and genetic approaches to identify the putative marrow-derived stem cells that give rise to breast cancer (5). A similar paradigm could be used to explain the stem cell origin of other cancers (6-14).

What we observed in this research is that there are stromal cells within the extracellular matrix of the breast cancer derived from the donor bone marrow. Although these cells are in the minority, they consist of macrophages, lymphocytes, fibroblasts, myofibroblasts and endothelial cells. We also observed that rarely stem cells of donor bone marrow origin transform to breast cancer within the breast. Cancer-promoting and cancer-initiating stem cells, while mainly residing in the organ of cancer origin, can also be derived from ectopic locations. Although we were successful in demonstrating that both cancer-promoting (stromal) and cancer-initiating (epithelial) stem cells can be derived from the bone marrow, we were not successful in demonstrating that non-transgenic bone marrow stem cells can reduce the incidence of breast cancer in mice transplanted with tagged normal bone marrow. These recipient transgenic mice exhibited the same temporal frequency and incidence of breast cancer as their non-transplanted controls. Conceivably the native transgenic stem cells of these mice present originally within the bone marrow may have already migrated to the breast prior to the bone marrow transplantation of normal non-transgenic stem cells. These transgenic cells derived from native bone marrow transform to breast cancer, an event which can not be prevented or reduced by non-transgenic bone marrow transplantation.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that stromal cells within the breast cancers that arise in transgenic mice contain significant percentages of tissue macrophages, lymphocytes, fibroblasts, myofibroblasts and endothelial cells, which presumably represent the progeny of cancer-promoting stem cells of donor origin (Figure 2B, 2C, 2D).
- Demonstration that these stromal cells of donor origin may affect breast cancer progression differently than those of endogenous breast origin.
- Demonstration that rare cancer-initiating stem cells of donor origin also give rise to breast cancer (Figure 2E).
- Demonstration that these ectopically-derived stem cells that transform to breast cancer may be different biologically compared to the endogenous breast stem cells that spontaneously transform to breast cancer.

REPORTABLE OUTCOMES

Abstracts and Presentations

Barsky SH, Xiao Y, Ye Y, Yearsley K. Mammary intraductal foam cells are bone marrow derived and are recruited in response to both physiological as well as neoplastic stimuli. Era of Hope 2008 Department of Defense Breast Cancer Research Program Meeting, 2008.

Barsky SH. Cancer-initiating stem cells that give rise to breast cancer can be both intrinsically as well as ectopically derived and can manifest an exaggerated stem cell phenotype within the lymphovascular embolus of both primary as well as secondary inflammatory breast cancer. Era of Hope 2008 Department of Defense Breast Cancer Research Program Meeting, 2008.

Barsky SH, Ye Y, Xiao Y, Yearsley K. Insights into the stem cell origin of human cancers by studying a registry of bone marrow and other organ transplant recipients who later developed solid cancers. (Oral Presentation). ASCO, 2008

Development of Animal Models

To achieve our first objective, we used two different murine female bitransgenic breast cancer models: 1) the highly penetrant though non-physiologic *MMTV-pymT* / ROSA26 transgenic where polyomavirus middle T antigen is under the control of the MMTV promoter and in which all of its tissues including bone marrow express the lacZ reporter; and 2) the less penetrant but more natural *MMTV-erbB2/neu* / ROSA26 bitransgenic. Marrow from each of the female bitransgenics harvested by femoral flushing, were tail vein-injected into sublethally irradiated normal healthy female mice as well as their respective unmarked transgenic recipients. In these latter recipient groups, both stromal cells and rarely carcinoma cells of donor origin were observed. These crosses represent developments of new hybrid animal models.

CONCLUSION

Cancer-promoting and initiating stem cells can be derived from ectopic locations in inflammatory and other breast cancers. Cancer-promoting and cancer-initiating stem cells, while mainly residing in the organ of cancer origin, can also be derived from ectopic locations. In a study of a registry of transplant recipients who had received sex-mismatched bone marrow and other organ transplants for various diseases and later developed secondary solid cancers including breast cancer, cancer-promoting stem cells giving rise to lymphocytes, fibroblasts, myofibroblasts, tissue macrophages and endothelial cells and rarely, cancer-initiating stem cells were observed within the secondary solid cancer that were of donor origin. Carrying forward these observational studies made in humans to testing these hypotheses experimentally in mouse models, we conducted bone marrow transplantations in transgenic mice genetically engineered to develop breast cancer. Being able to mark the donor bone marrow enzymatically, we were able to study the breast cancers that developed in the mammary gland for the presence of stem cells derived from the bone marrow. Using either the bitransgenic *MMTV-pymT/ROSA26* or the *MMTV-erbB2/neu/ROSA26* models as donors and either lethally irradiated wild type or single unmarked transgenics as recipients, we were able to demonstrate that the breast cancers that emerged contained significant percentages of tissue macrophages, lymphocytes, fibroblasts, myofibroblasts and endothelial cells, which presumably represented the progeny of cancer-promoting stem cells of donor origin. Rare cancer-initiating stem cells of donor origin were also observed. These ectopically-derived cells may affect breast cancer progression differently than those of endogenous breast origin.

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APPENDICES

Abstracts and Presentations

1. **Barsky SH**, Xiao Y, Ye Y, Yearsley K. Mammary intraductal foam cells are bone marrow derived and are recruited in response to both physiological as well as neoplastic stimuli. Era of Hope 2008 Department of Defense Breast Cancer Research Program Meeting, 2008.

2. **Barsky SH**. Cancer-promoting and initiating stem cells can be derived from ectopic locations in inflammatory and other breast cancers. Era of Hope 2008 Department of Defense Breast Cancer Research Program Meeting, 2008.

3. **Barsky SH**, Ye Y, Xiao Y, Yearsley K. Insights into the stem cell origin of human cancers by studying a registry of bone marrow and other organ transplant recipients who later developed solid cancers.(Oral Presentation). ASCO, 2008

1.
MAMMARY INTRADUCTAL FOAM CELLS ARE BONE MARROW DERIVED AND ARE RECRUITED IN RESPONSE TO BOTH PHYSIOLOGICAL AS WELL AS NEOPLASTIC STIMULI

Sanford H. Barsky, Yi Xiao, Yin Ye, Kurtis Yearsley

Intraductal "foam cells" are the most commonly encountered cells in spontaneous nipple discharge, nipple aspirate fluid and ductal lavage yet their origin and significance remain a mystery. These cells increase in pregnancy and other conditions of ductal ectasia and obstruction. They frequently surround DCIS and other intraductal proliferations. Our previous immunocytochemical studies with macrophage (CD68, lysozyme), epithelial (cytokeratin, estrogen receptor) and myoepithelial (smooth muscle actin, CALLA, maspin) markers indicated that foam cells are of macrophage lineage and terminally differentiated (negative Ki-67 and PCNA). These foam cells often ingest both endogenous as well as exogenous substances. Because these macrophages are observed only intraductally and because their appearance resembles lactating and vacuolated epithelial cells, their origin had been presumed to be of ductal lining epithelium. However our previous studies utilizing bone marrow transplantation of donor marrow from female GFP-transgenic C57 black mice into sublethally irradiated female C57 black mice recipients rendered pseudopregnant revealed that the mammary foam cells were of donor origin. Mice exhibiting successful bone marrow engraftment of at least 50% donor marrow were identified and made pseudopregnant with a combination of estradiol, progesterone and estriol (2.5mg) 21 day release pellets. After this time period and following euthanasia, their mammary fat pads were excised and examined. The presence of GFP - containing intraductal foam cells was found. As controls for nonspecific phagocytosis of GFP, no free GFP was found within ductal fluid. Furthermore tail vein injections of GFP- labelled murine lymphocytes and embryonal fibroblasts into female C57 mice made pseudopregnant produced no GFP - containing mammary foam cells. We have extended these initial studies to donor ROSA26 transgenic mice containing the lacZ reporter gene and recipient transgenic mice carrying potent breast cancer oncogenes: 1) the highly penetrant very robust though somewhat unnatural *MMTV-pymT* where polyomavirus middle T is overexpressed and where 100% of mice develop breast cancer with a mean onset of 90 days and 2) the less

penetrant but more natural *MMTV-erbB2/neu*. Marrow from each of the ROSA26 donors was transplanted into three transgenic recipients containing either of the aforementioned oncogenes or wild type controls. Sub-groups of recipients having a successful marrow take were rendered pseudopregnant with subcutaneous pellets of estradiol (0.5 mg), progesterone (25 mg) and estriol (2.5mg). In both the pseudopregant as well as non-pregnant oncogene transgenic recipients as well as controls, the mammary foam cells were of donor origin. However the number of intraductal foam cells were increased in pseudopregnancy 10 fold, by intraductal neoplasia 5 fold and by a combination of the two over 25 fold. Ducts containing neoplastic cells with the highest numbers of mammary foam cells exhibited a significantly increased apoptotic index of the neoplastic cells by TUNEL. These findings suggest a new strategy of delivering therapeutic genes to DCIS, other precancerous lesions or high risk ductal epithelium a strategy which would exploit the omnipresent mammary foam cell, its bone marrow origin and its chemoattraction to the breast in response to both physiological as well as neoplastic stimuli.

2.

CANCER-PROMOTING AND INITIATING STEM CELLS CAN BE DERIVED FROM ECTOPIC LOCATIONS IN INFLAMMATORY AND OTHER BREAST CANCERS

Sanford H. Barsky, Yi Xiao, Yin Ye, Kurtis Yearsley

Although human breast cancer is all too common, circumstantial evidence exists on a cellular level to suggest that cancer transformation is a rare event. Even in the setting of inherited breast cancer, eg. BRCA1 when all the cells of the breast contain the inherited BRCA1 mutations, transformation on a cellular level is still rare. This suggests that only certain cells are capable of cancer initiation and promotion. Cancer-promoting and cancer-initiating stem cells, while mainly residing in the organ of cancer origin, can also be derived from ectopic locations. In a study of a registry of transplant recipients who had received sex-mismatched bone marrow and other organ transplants for various diseases and later developed secondary solid cancers including breast cancer, cancer-promoting stem cells giving rise to lymphocytes, fibroblasts, myofibroblasts, tissue macrophages and endothelial cells and rarely, cancer-initiating stem cells were observed within the secondary solid cancer that were of donor origin. Carrying forward these observational studies made in humans to testing these hypotheses experimentally in mouse models, we conducted bone marrow transplantations in transgenic mice genetically engineered to develop breast cancer. Being able to mark the donor bone marrow enzymatically, we were able to study the breast cancers that developed in the mammary gland for the presence of stem cells derived from the bone marrow. Using either the bitransgenic *MMTV-pymT/ROSA26* or the *MMTV-erbB2/neu/ROSA26* models as donors and either lethally irradiated wild type or single unmarked transgenics as recipients, we were able to demonstrate that the breast cancers that emerged contained significant percentages of tissue macrophages, lymphocytes, fibroblasts, myofibroblasts and endothelial cells, which presumably represented the progeny of cancer-promoting stem cells of donor origin. Rare cancer-initiating stem cells of donor origin were also observed. These ectopically-derived cells may affect breast cancer progression differently than those of endogenous breast origin. In another experimental model system, we used a human xenograft of inflammatory breast cancer (MARY-X) which demonstrated, as in patients, florid lymphovascular tumor emboli within lymphovascular channels. These emboli which were resistant to chemotherapy, exhibited a prominent stem cell-like phenotype with high expression of

CD133, CD44, ALDH1, oct-4, nanog, sox-2, stellar, rex-1, H19 and nestin, suggesting that the lymphovascular tumor emboli, like the human embryonal blastocyst, are derived from stem cells locked in self-renewal. In parallel bone marrow transplant experiments, some of the endothelial cells which lined the channels containing the tumor emboli exhibited evidence of bone marrow origin. Inflammatory breast cancer in humans oftentimes consists of only florid lymphovascular tumor emboli without the presence of a pre-cancerous or invasive cancerous mass in the breast and therefore may reflect the presence of both cancer-promoting as well as cancer-initiating stem cells derived from ectopic locations.

3.

INSIGHTS INTO THE STEM CELL ORIGIN OF HUMAN CANCERS BY STUDYING A REGISTRY OF BONE MARROW AND OTHER ORGAN TRANSPLANT RECIPIENTS WHO LATER DEVELOPED SOLID CANCERS

Sanford H. Barsky, Yi Xiao, Yin Ye, Kurtis Yearsley

Background: The existence of cancer stem cells (CSCs) in solid cancers has been presumed based on the properties of self-renewal, multipotency and embryonal markers exhibited by tumor subpopulations in vitro. But the in vivo existence for CSCs is still unproven. Because of isolated reports of tumors of donor origin arising in transplant recipients and because of the stem cell implications of this finding, we studied a registry of transplant recipients who later developed solid cancers to investigate this question.

Methods: We studied 165 renal, 25 heart, 25 liver, 10 lung and 55 bone marrow transplant recipients. The solid cancers arising in these patients included skin, soft tissue, lung, liver, kidney, breast and others. 48% of these transplants were from different sex donors. We created a tissue microarray (TMA) consisting of the secondary solid cancers, adjacent normal tissues and control cancers from non-transplant patients. We conducted X and Y chromosome FISH and, in selected cases, microsatellite marker, ploidy and gene rearrangement studies.

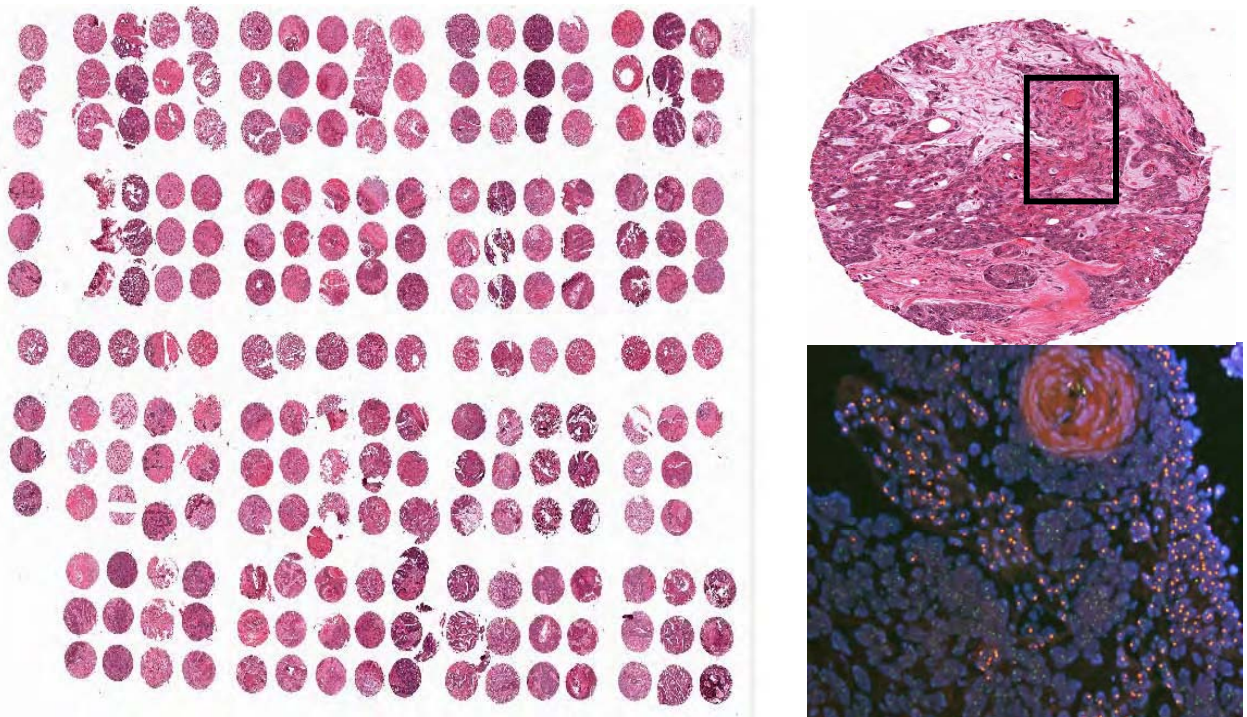
Results: Approximately 12% of the solid cancers arising in non-sex matched transplant recipients were of donor origin. The vast majority of these were in patients receiving a bone marrow transplant and were secondary cancers. This was seen in both female as well as male recipients. The numbers may actually have been higher than observed because some cancers of male origin spontaneously lost the Y chromosome. Conversely in some donor organs where primary solid cancers arose, the cancers were recipient in origin in 5% of the cases. In selected cases, microsatellite marker studies confirmed the donor or recipient origin. Ploidy and gene rearrangement studies excluded lymphocytic fusion or donor-recipient chimerism as mechanisms to explain the findings.

Conclusions: Our studies suggest that solid cancers arising in transplant recipients can take origin from stem cells that are derived from either the transplanted organ (usually bone marrow) or the host. In either case these stem cells do not initially reside in the organ where the cancer developed. These observations could explain the clinical properties of solid cancers: rareness of transformation, tumor dormancy, local recurrence and distal metastasis.

SUPPORTING DATA

(See following pages)

Figure 1: We initially conducted XY chromosome FISH



In a study of a registry of transplant recipients who had received sex-mismatched bone marrow and other organ transplants for various diseases and later developed secondary solid cancers including breast cancer, cancer-promoting stem cells giving rise to lymphocytes, fibroblasts, myofibroblasts, tissue macrophages and endothelial cells and rarely, cancer-initiating stem cells were observed within the secondary solid cancer that were of donor origin.

HYPOTHESES OF ECTOPIC ORIGIN OF CANCER-PROMOTING (CPCs) AND CANCER-INITIATING (CICs) STEM

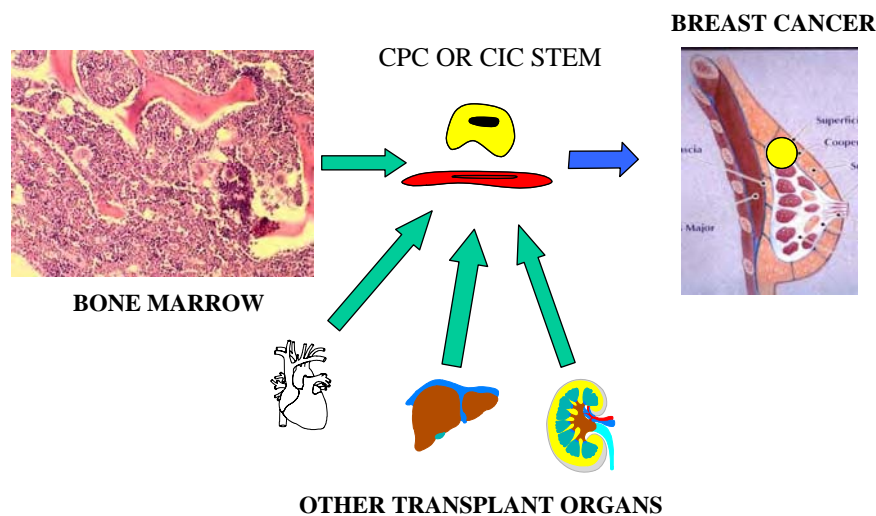
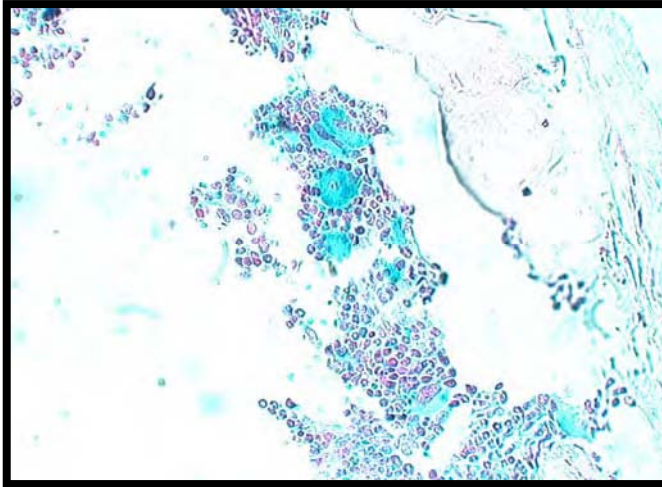
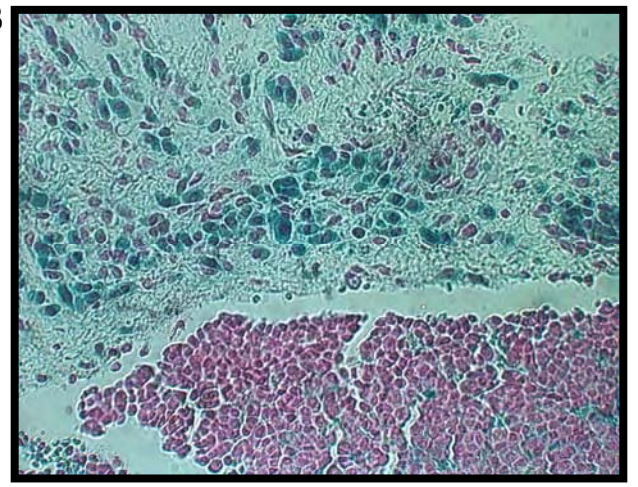


Figure 2

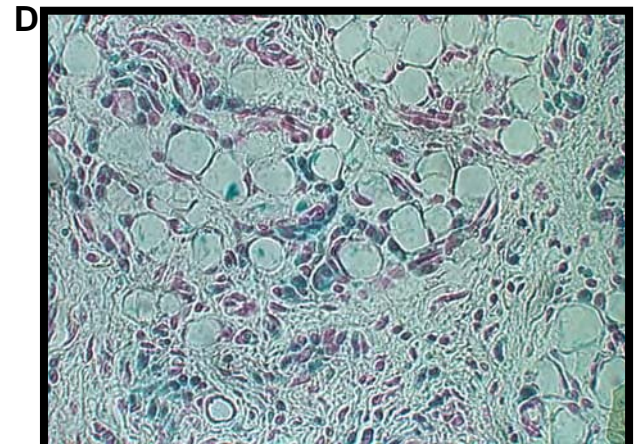
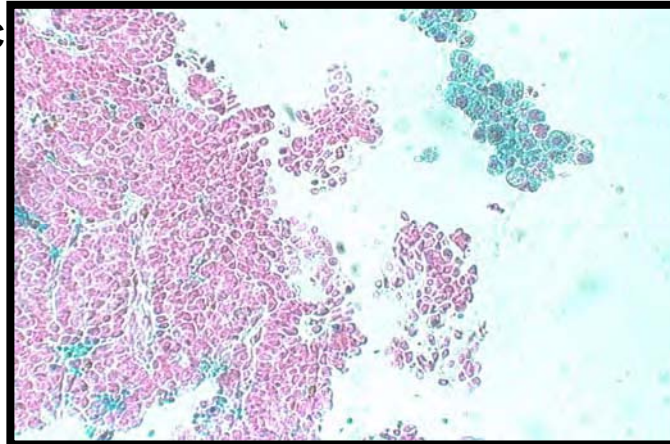
A



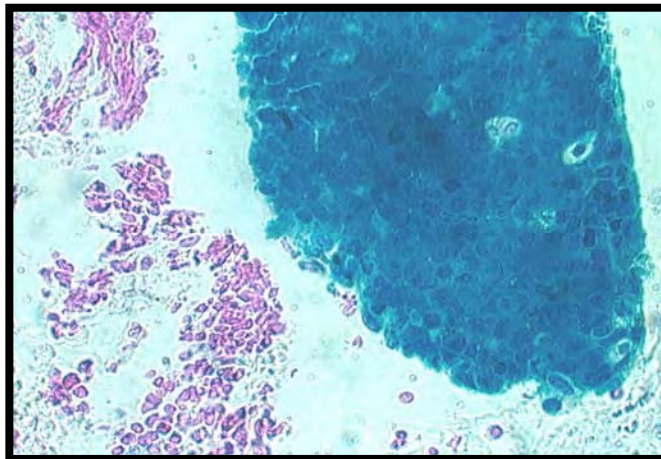
B



C



E



Being able to mark the donor bone marrow enzymatically with LACZ (blue) (A), we were able to study the breast cancers that developed in the mammary gland for the presence of stem cells derived from the bone marrow. Using either the bitransgenic *MMTV-pymT/ROSA26* or the *MMTV-erbB2/neu/ROSA26* models as donors and either lethally irradiated wild type or single unmarked transgenics as recipients, we were able to demonstrate that the breast cancers that emerged contained significant percentages of lymphocytes, fibroblasts, myofibroblasts (B), tissue macrophages (C) and tissue endothelial cells (D), which presumably represented the progeny of cancer-promoting stem cells of *donor* origin. Rare cancer-initiating stem cells of *donor* origin were also observed (E). These ectopically-derived cells may affect breast cancer progression differently than those of endogenous breast origin.